this part of the genomic sequence and we have not identified a unique primer extension or 5′ rapid amplification of cloned ends (RACE) product. However, we have cloned the presumptive 3′ ends of the *n* and *b* transcripts using 3′ RACE. The *Fv1*^{n} allele of AKR terminates at the R-U5 boundary of the 5′ long terminal repeat (LTR) of the inserted IAP, whereas the *b* allele is polyadenylated within the second B2 element downstream of the ORF. The *Fv1* mRNA is unusual, therefore, in having a single large exon, encoding a protein of ~50K and transcripts terminating within insertion elements found downstream of the ORF.

Sequence homology was detected with two expressed sequence tags (accession numbers X71645 and R99971), as well as a genomic clone (X89211) representing a member of the human endogenous retrovirus family, HERV-L[16] (Fig. 4*a*). *Fv1* and HERV-L show 60% identity in the 5′ region of the presumptive *gag* gene of HERV-L. Hybridization between *Fv1* and the HERV-L provirus, which is present at 200 copies per haploid genome equivalent in human and mouse, but at a much lower level in rat[16], may explain the background smear seen in low-stringency Southern blots. Thus, *Fv1* is likely to be derived from a retrovirus of the HERV-L family.

Fv1 and the endogenous human retrovirus HERV-L are 60% identical over a stretch of 1,300 base pairs. This region of the cloned HERV-L does not encode an ORF, presumably reflecting mutational decay of the endogenous retrovirus, and shows no discernible sequence homology to other retroviruses. But based on its position between an LTR and a *pol* gene, it seems that it corresponds to the *gag* gene of the HERV-L family. The Fv1 protein may therefore be Gag-like and share some structural similarity with its target, the CA protein of MLV. Although this presumed similarity in *gag* might be important for virus restriction, for example to permit binding of Fv1 to CA, there are hundreds of retroviral elements more closely related to MLV than *Fv1* and none of these restricts replication.

To study the evolution of *Fv1* further we examined DNAs from a variety of mammals. Single-copy bands were seen in samples of inbred mice and in *Mus dunni* (Fig. 4*b*), but no reactive bands were seen in samples from rat, cat and human. The *Fv1* gene is therefore present only in mice, or it shows a high degree of sequence divergence even between mouse and rat. The overall similarity of the *Fv1* locus in *Mus dunni* (not shown) and *Mus musculus* (C57BL/6) mice implies that the original integration event preceded the divergence of the progenitors of the two species and that the *Fv1* gene is likely to be widely distributed among *Mus* species. Endogenous MLVs are less widely distributed[17]; they appear to have colonized the *Mus* germ line more recently. *Fv1* activity is found only in strains containing endogenous MLVs[18], suggesting that the antiviral activity may have evolved in response to the presence of MLVs. The apparently still-more-recent generation of the *Fv1*^{n} allele suggests that this may be an on-going process. Given the widespread distribution of retroviral elements, it is perhaps not surprising that genes from these elements can be co-opted for the purposes of the host, for example in protecting against retroviral infection[19]. The demonstration of such activities in mice raises the question of whether analogous restriction systems have evolved elsewhere, for instance in humans, where the absence of inbred strains would have obscured their presence. □

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The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry

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CHEMOKINES are chemotactic cytokines that activate and direct the migration of leukocytes[1,2]. There are two subfamilies, the CXC and the CC chemokines. We recently found that the CXC-chemokine stromal cell-derived factor-1 (SDF-1)[3,4] is a highly efficacious lymphocyte chemoattractant[5]. Chemokines act on responsive leukocyte subsets through G-protein-coupled seven-transmembrane receptors[1], which are also used by distinct strains of HIV-1 as cofactors for viral entry. Laboratory-adapted and some T-cell-line-tropic (T-tropic) primary viruses use the orphan chemokine receptor LESTR/fusin (also known as fusin)[6–8], whereas macrophage-tropic primary HIV-1 isolates use CCR-5 and CCR-3 (refs 7–11), which are receptors for known CC chemokines. Testing of potential receptors demonstrated that SDF-1 signalled through, and hence 'adopted', the orphan receptor LESTR, which we therefore designate CXC-chemokine receptor-4 (CXCR-4). SDF-1 induced an increase in intracellular free Ca^{2+} and chemotaxis in CXCR-4-transfected cells. Because SDF-1 is a biological ligand for the HIV-1 entry cofactor LESTR, we tested whether it inhibited HIV-1. SDF-1 inhibited infection by T-tropic HIV-1 of HeLa-CD4 cells, CXCR-4 transfectants, and peripheral blood mononuclear cells (PBMCs), but did not affect CCR-5-mediated infection by macrophage-tropic (M-tropic) and dual-tropic primary HIV-1.

The chemokine SDF-1 has multiple biological activities and may be a primordial chemokine. It was initially defined by cloning as a bone-marrow stromal cell-derived factor, and as a pre-B-cell stimulatory factor[3,4]. We recently isolated SDF-1 as a T-lymphocyte chemoattractant, and found it to be active on monocytes but not neutrophils[5]. Up to 80% of resting T lymphocytes respond to SDF-1, as opposed to 1–10% for other chemokines, suggesting that its receptor is either highly active or well expressed on resting T lymphocytes. All other known CXC- and CC-chemokines cluster on chromosomes 4 and 17, respectively, but the SDF-1 gene is on chromosome 10 (ref. 12). Sequence homologies show that SDF-1 is related almost equally to both CC- and CXC-chemokines forming an outgroup, and is predicted to have diverged little from a primordial CC- and CXC-chemokine ancestor. Consistent with this, SDF-1 is unusually well conserved between species, with a single substitution of Ile to Val between mouse and human[12]. SDF-1α and SDF-1β arise by differential splicing from a single gene, and differ in four carboxy-terminal amino acids that are present in SDF-1β but absent in SDF-1α.

We isolated two forms of murine SDF-1 from stromal cells that appear to be processed relative to SDF-1α, as predicted from the cDNA, one of which lacks the five N-terminal amino-acid residues[5]. We synthesized and refolded the corresponding human proteins, the longer SDF-1α (residues 1–67) and the shorter SDF-1α (6–67). To test for biological activity of the synthetic proteins, we performed chemotaxis assays[5,13] with freshly prepared human peripheral blood lymphocytes using Transwell inserts. This allows the exact quantification of migrated versus non-migrated cells[5,13], by counting the cells that transmigrate through the filter and drop into the bottom chamber. Full-length SDF-1α (1–67) induced maximal lymphocyte chemotaxis at a concentration of 1 μg ml^{-1} and was identical in activity to purified natural murine SDF-1α (Fig. 1). In contrast, SDF-1α (6–67) was inactive up to a concentration of 3.3 μg ml^{-1}. This shows that the N-terminal amino acids are important for SDF-1 function, as has previously been shown for other chemokines[1].

To identify a receptor that binds SDF-1, we screened chemokine receptor-like orphans for their ability to elicit increases in intracellular Ca^{2+} in response to SDF-1. CHO cells stably transfected with human LESTR[14–18] consistently showed increases in intracellular Ca^{2+} when full-length SDF-1α (1–67) was added. Ca^{2+} fluxes were seen with SDF-1 concentrations ranging from 1 μg ml^{-1} (Fig. 2*a*) to 100 ng ml^{-1} (data not shown). In contrast, untransfected cells were unresponsive to SDF-1 (Fig. 2*b*). Addition of SDF-1α (1–67) to LESTR-transfectants abrogated responsiveness to a subsequent addition of SDF-1α (1–67), demonstrating homologous desensitization (Fig. 2*a*). Consistent with earlier negative functional data[18], addition of RANTES (regulated on activation normal T cell expressed and secreted), monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-8 to a final concentration of 1 μg ml^{-1} did not induce a response. These chemokines also did not desensitize cells to SDF-1α (1–67) (Fig. 2*c*). LESTR-transfected CHO cells were unresponsive to addition of N-terminally truncated SDF-1α (6–67) (Fig. 2*d*). Furthermore, SDF-1α (6–67) did not antagonize a response to subsequent stimulation with full-length SDF-1α (1–67), suggesting that the truncated protein either does not bind LESTR or has a substantially lower affinity than SDF-1α (1–67). The Ca^{2+} flux assay was confirmed with a modified Boyden-chamber chemotaxis assay. LESTR-transfected CHO cells migrated specifically in response to SDF-1α (1–67) (Fig. 2*e*), whereas untransfected cells did not show migration above background (data not shown).

Chemokines and their receptors have recently been shown to play critical roles in early events in HIV-1 infection. The chemokine receptor-like orphan LESTR was identified as a necessary cofactor for entry of T-tropic laboratory-adapted[6] and T-tropic primary[7,8] HIV-1 strains into $CD4^+$ cells. Having identified SDF-1 as a biological ligand for LESTR, we hypothesized that it would interfere with use of this receptor by HIV-1. To test for inhibition

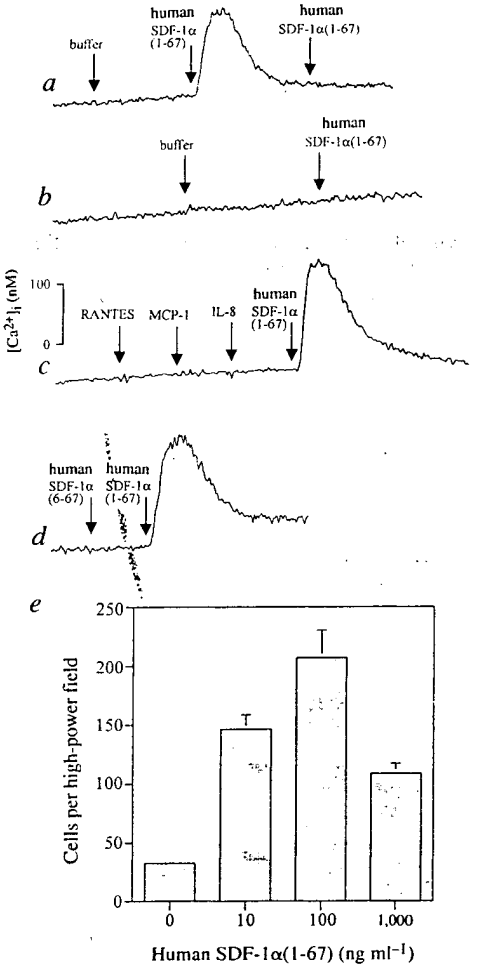

FIG. 2 Mobilization of Ca^{2+} and chemotaxis of LESTR-transfected CHO cells in response to full-length SDF-1α (1–67) and truncated SDF-1α (6–67). *a–d*, A stable LESTR-transfected CHO cell line (*a*, *c*, *d*) and untransfected CHO cells (*b*) were loaded with Fura-2 AM fluorescent dye, and increases in intracellular Ca^{2+} on addition of the indicated chemokines were measured in a fluorescence spectrophotometer. SDF-1α (1–67), SDF-1α (6–67), RANTES, MCP-1 and IL-8 were added to a final concentration of 1 μg ml^{-1}. *e*, Chemotactic activity of SDF-1α (1–67) in a modified Boyden-chamber chemotaxis assay for a LESTR-transfected CHO cell line. Error bars indicate s.d. of quadruplicates.

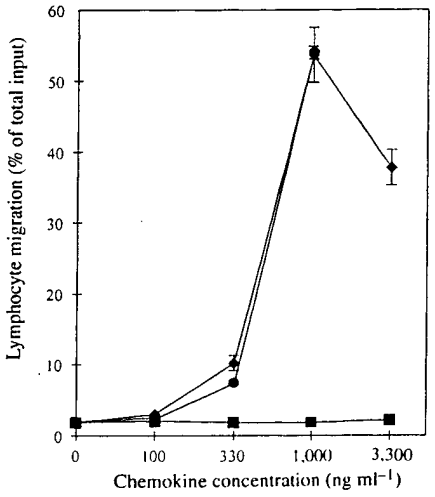

FIG. 1 Chemotactic activity of synthetic human SDF-1α (1–67) (diamonds), SDF-1α (6–67) (squares) and purified murine SDF-1α (circles) on freshly purified human peripheral blood lymphocytes (PBLs). PBLs were migrated through 5-μm pore Transwell inserts for 3 h. Error bars indicate the range of duplicates. One of three experiments is shown.

by SDF-1 of entry of T-tropic HIV-1 strains into permissive cells, we used an *env*-complementation assay[7,19], in which HIV-1 envelope glycoproteins expressed in *trans* complement a single round of replication of an *env*-deleted provirus encoding the chloramphenicol acetyltransferase (CAT) gene. We produced recombinant virus that was pseudotyped with the envelope glycoproteins of the laboratory-adapted HXBc2 isolate, which uses LESTR for fusion[7]. Because HeLa cells are known to express LESTR at high levels[6], we incubated HeLa-CD4 cells, which express human CD4 in the presence of either SDF-1α (1–67) or SDF-1α (6–67), with recombinant viruses containing the HXBc2 envelope glycoproteins. The efficiency of the early phase of virus infection was assessed by measuring CAT activity in the target cells 60 h after infection. Full-length SDF-1α (1–67) completely inhibited infection of the recombinant viruses in a concentration-dependent manner, whereas the truncated SDF-1α (6–67) showed no inhibition (Fig. 3*a*).

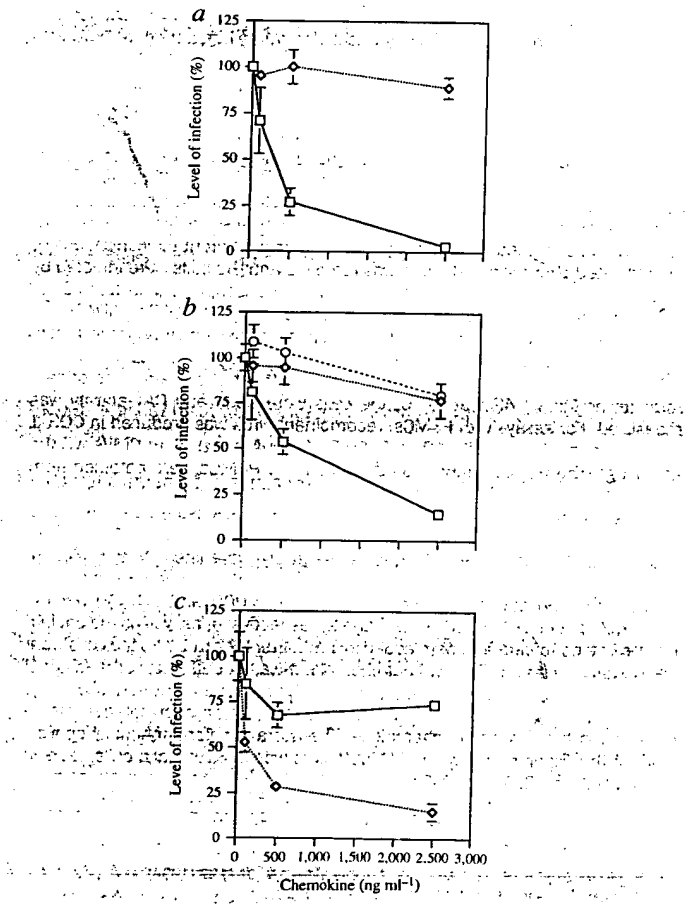

FIG. 3 Inhibition of LESTR-mediated infection of recombinant HIV-1 by full-length SDF-1α (1–67). *a*, HeLa-CD4 cells expressing human CD4 were incubated with recombinant HIV-1 virus pseudotyped with the envelope glycoproteins of the laboratory-adapted HXBc2 isolate in the presence of different concentrations of full-length SDF-1α (1–67) (squares) and N-terminally truncated SDF-1α (6–67) (diamonds)[7,19]. Results are indicated as percentage of chloramphenicol conversion observed in the absence of chemokine. Results with s.d. of three independent experiments are shown. *b*, Cf2Th-CD4 canine thymocytes transfected with either LESTR (squares, diamonds) or CCR-5 (circles) were incubated with recombinant HIV-1 virus pseudotyped with the envelope glycoproteins of either HXBc2- (diamonds, squares) or ADA- (circles) isolates. Full-length SDF-1α (1–67) (squares, circles) and truncated SDF-1α (6–67) (diamonds) were added at the indicated concentrations. Error bars show the range of duplicate experiments. *c*, Cf2Th-CD4 canine thymocytes transfected with either LESTR (diamonds) or CCR-5 (squares) were incubated in the presence of the indicated concentrations of SDF-1α (1–67) with recombinant HIV-1 virus pseudotyped with the envelope glycoproteins of the dual-tropic primary HIV-1 isolate 89.6. Error bars show the range of duplicate experiments.

To demonstrate that the HIV-1-inhibitory effect of SDF-1 was mediated through interaction with LESTR, we used Cf2Th canine thymocytes as target cells. Cf2Th cells are non-permissive for HIV-1 infection unless transfected with plasmids expressing human CD4 and chemokine receptors specific for particular viral strains[7]. Cf2Th canine thymocytes expressing human CD4 and LESTR were incubated with recombinant virus containing HXBc2 envelope glycoproteins in the presence of either SDF-1α (1–67) or SDF-1α (6–67). SDF-1α (1–67) effectively inhibited infection, whereas truncated SDF-1α (6–67) had no effect (Fig. 3*b*). Furthermore, SDF-1α (1–67) did not inhibit infection of Cf2Th cells expressing CD4 and CCR-5 by a recombinant HIV-1 virus containing the envelope glycoproteins of the M-tropic primary HIV-1 strain ADA (Fig. 3*b*). These data suggest that functional SDF-1 effectively inhibits entry of HIV-1 virus using LESTR without interfering with infection by HIV-1 strains using CCR-5.

To exclude the possibility that SDF-1 mediated its virus-inhibitory effect by interacting with the viral envelope glycoproteins, we tested recombinant HIV-1 pseudotyped with the envelope glycoproteins of the dual-tropic primary HIV-1 strain 89.6, which is known to use the chemokine receptors LESTR, CCR-5 and CCR-3 for fusion[7,8]. Full-length SDF-1α (1–67) inhibited infection by the 89.6 virus of canine thymocytes expressing LESTR, but there was little effect on infection of CCR-5-transfected cells (Fig. 3*c*). These findings indicate that the HIV-1 inhibitory effect of SDF-1 is specific for LESTR. Furthermore, the experiments demonstrate that SDF-1 inhibits not only laboratory-adapted strains but also primary HIV-1 isolates that use LESTR for entry into permissive cells.

To determine whether SDF-1 would inhibit infection of primary cells, peripheral blood mononuclear cells (PBMCs) were used as target cells in the *env*-complementation assay. Full-length SDF-1α (1–67), but not truncated SDF-1α (6–67), inhibited the early phase of PBMC infection by virus pseudotyped with the envelope of the HXBc2 HIV-1 strain (Fig. 4).

Our search for the receptor for SDF-1 surprisingly led to the same orphan receptor as had been identified to mediate fusion of T-tropic HIV-1 strains with CD4-bearing cells[6]. We not only provide evidence that SDF-1 is a biological ligand for LESTR, but also that it specifically inhibits LESTR-mediated infection by both laboratory-adapted as well as primary T-tropic HIV-1 strains. Moreover, the latter experiments support the evidence that SDF-1 binds LESTR. Now that this receptor has been found to bind a CXC-chemokine, it can be designated CXC-chemokine receptor-4 (CXCR-4). The IL-8 receptor A and B and a recently described receptor for the monokine induced by gamma interferon (Mig) and the interferon-inducible protein 10 (IP-10) (ref. 20) represent the other members of the CXC-chemokine receptor family.

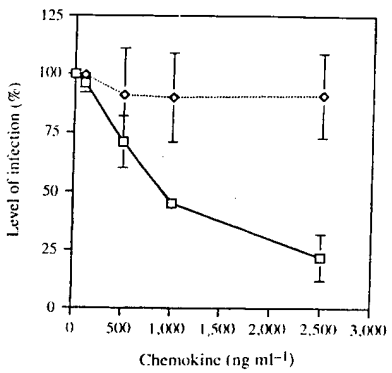

FIG. 4 Inhibition by SDF-1α (1–67) of infection of PBMCs by recombinant HIV-1. PHA-stimulated PBMCs were incubated with CAT-expressing recombinant HIV-1 virus pseudotyped with the HXBc2 envelope in the presence of different concentrations of full-length SDF-1α (1–67) (squares) and N-terminally truncated SDF-1α (6–67) (diamonds). CAT activity was measured 60 h after infection. Results from duplicate wells of two independent experiments are indicated as percentage of chloramphenicol conversion observed in the absence of chemokine.

Although it is intriguing that SDF-1 is the most efficacious chemoattractant for T lymphocytes identified so far, and its receptor is fusogenic for T-tropic HIV-1 isolates, both the functional importance of SDF-1 and the cell distribution of CXCR-4 are much broader. SDF-1 is chemoattractive for B lymphocytes (C.C.B. and T.A.S., unpublished data), is a pre-B-cell stimulatory factor[4], and is chemoattractive for haematopoietic stem cells (A. Aiuti, I. J. Webb, C.C.B., T.A.S. and J. C. Gutierrez-Ramos, in preparation). In agreement with this, mice genetically deficient in SDF-1 lack B cells and have myelopoiesis in the fetal liver but not in the bone marrow[21]. SDF-1 mRNA is well expressed in many non-haematopoietic tissues including the heart, brain, lung, kidney and liver[3,12]. Similarly, CXCR-4 mRNA is well expressed in non-haematopoietic tissues including the brain, heart, kidney and lung[15,22]; indeed, CXCR-4 is by far the most widely expressed of the functional chemokine receptors in non-haematopoietic cells. An important function for SDF-1 in non-haematopoietic cells is illustrated by perinatal lethality and failure of the ventricular septum of the heart to close in animals genetically deficient in SDF-1 (ref. 21). The high conservation of SDF-1 is mirrored in its receptor, which is 93% identical between human and bovine CXCR-4 (refs 8, 22). Because of the multiple biological functions, it will be important to determine whether SDF-1 binds to receptors other than CXCR-4, or whether there are additional ligands for CXCR-4.

Both the fusogenic receptors and their biological ligands are now known for most strains of HIV-1. M-tropic HIV-1 isolates use the chemokine receptors CCR-5 and CCR-3 as cofactors for membrane fusion[7–11]. Chemokines that bind CCR-5 and CCR-3 have been shown to inhibit entry of primary M-tropic but not T-tropic HIV strains[7,10,23]. Similarly, we have shown here that entry of a T-tropic strain that uses CXCR-4 is inhibited by the ligand SDF-1. Some primary HIV-1 isolates are T-tropic and use CXCR-4 (refs 7, 8), but most are M-tropic and use CCR-5 (refs 7–11); some primary isolates also use CCR-3 (refs 7, 8). Monocytes respond chemotactically to SDF-1 just as well as do T lymphocytes[5], and express mRNA for CXCR-4 (ref. 18), so factors in the virus–host relationship other than ability to infect monocytes may determine the use of CCR-5 by most primary isolates. T-tropic HIV-1 strains can be found in HIV-1-infected individuals late in the course of infection. They induce the formation of syncytia in $CD4^+$ cell lines, infect PBMCs faster, and replicate in these cells at a higher rate than M-tropic primary isolates[24,25]. Occurrence of T-tropic primary HIV isolates in infected individuals is associated with $CD4^+$ cell decline and progression to AIDS, and may suggest a detrimental role of these isolates in AIDS pathogenesis. The finding that SDF-1 attracts far more T lymphocytes than other chemokines[5] suggests that CXCR-4 may be an abundant receptor on T cells, and may explain the use of this fusogenic receptor by T-tropic HIV-1. Why receptors for chemokines have been subverted by HIV-1 to trigger cell fusion and the molecular mechanisms that trigger fusion are intriguing subjects. The chemokines that bind these receptors will be important tools for HIV research; whether the chemokines, or antagonists of chemokine receptors, will prove useful in the fight against HIV is an important topic for further research. □

Methods

Chemokines and chemotaxis assay. Murine SDF-1α was purified from supernatants of the murine bone-marrow stromal cell line MS-5 (ref. 5). Synthetic human SDF-1α (1–67) and SDF-1α (6–67) were generated using tBoc chemistry on an Applied Biosystems 430A peptide synthesizer[26]. Proteins were folded by air oxidation and purified using at least two steps of reverse-phase high-performance liquid chromatography (RP–HPLC)[26]. The molecular mass for each protein was determined by electrospray mass spectrometry on a API-3 triple quadrupole mass spectrometer (SCIEX, Thornhill, Ontario). The average masses ± s.d., with the calculated mass minus the measured mass in parentheses, were SDF-1α (1–67) 7,831.63 ± 0.76 (−0.32) and SDF-1α (6–67) 7,306.39 ± 0.45 (0.25). Lymphocyte chemotaxis assays were performed as described[5]. Human peripheral blood lymphocytes were obtained from healthy donors by Ficoll-Histopaque separation. Monocytes were removed by two 30-min steps of plastic adherence. Cells (5×10^5) in 100 μl RPMI-1640 medium, 0.25% human serum albumin (HSA), were added to the top chamber of a 5-μm pore polycarbonate Transwell culture insert (Costar, Cambridge, MA) and incubated with the indicated concentrations of protein for 3 h. Transmigrated cells were counted with a FACScan (Becton Dickinson, San Jose, CA) using scattergates for lymphocytes for 20 s at 60 μl min^{-1}. For chemotaxis of LESTR-transfected CHO cells, a modified Boyden-chamber chemotaxis assay was used[27]. LESTR-transfected CHO cells were resuspended in RPMI 1640 medium containing 0.25% HSA and added in quadruplicate (15×10^3 cells per well) on top of a PVP-free, 8-μm pore polycarbonate membrane previously coated with 15 μg ml^{-1} fibronectin/PBS (Sigma) in a microchemotaxis chamber (Neuro Probe, Cabin John, MD). Transmigrated cells were stained and cells visible in a ×20 high-power field were counted.

Calcium fluorimetry. Untransfected CHO cells and the stable LESTR-transfected CHO cell line 1C2 were grown as described[18]. For Ca^{2+}-mobilization studies, 10^7 cells were incubated in 1 ml loading buffer containing 136 mM NaCl, 48 mM KCl, 1 mM $CaCl_2$, 1 mg ml^{-1} glucose and 20 mM HEPES, pH 7.4, with 5 nmol Fura-2 AM (Molecular Probes, Eugene, OR) for 20 min at 37 °C. The loaded cells were centrifuged, resuspended in fresh loading buffer, added to a stirred cuvette (2×10^6 in 500 μl) and inserted into a Hitachi F2000 spectrometer. Chemokines were added in a volume of 5 μl to a final concentration of 1 μg ml^{-1} at the indicated time points. Intracellular calcium concentrations were calculated using the Hitachi F2000 Intracellular Cation Measurement Program.

Envelope-complementation assay. Envelope-complementation assays were performed as described earlier[7,19]. Recombinant virus was produced in HeLa cells by cotransfection with 15 μg pHXBH10ΔenvCAT and 3 μg pSVIIIenv. HeLa-CD4 cells (clone 1022) were obtained form the NIH AIDS Research and Reference Reagent Program. Cf2Th canine thymocytes were transfected in duplicate by the calcium phosphate method with 10 μg of pCD4 plasmid and 25 μg of pcDNA3 expressing either LESTR or CCR-5, and were used approximately 60 h after transfection[7]. Approximately 8 h after replating, cells were incubated in 1 ml medium for 90 min at 37 °C with different concentrations of the indicated chemokine. Medium was removed and the cells were infected by incubation with recombinant virus (5,000 reverse transcriptase units for HXBc2-HeLa experiments and 20,000 reverse transcriptase units for HXBc2/ADA/89.6-Cf2Th experiments) pseudotyped with the respective envelope proteins in 1 ml of medium containing the original chemokine concentration. After 12 h at 37 °C, the medium was removed and fresh medium without chemokine was added. After an additional 48 h at 37 °C, the cells were lysed and CAT activity was measured. For assays with PBMCs, recombinant virus was produced in COS-1 cells by cotransfection with 10 μg CMVΔP1ΔenvpAvpu/vp, 5 μg CMVenvΔXho and 7 μg v653 RtatpC (HXBc2) as described[28,29]. PBMCs were obtained from healthy donors by Ficoll-Hypaque separation and grown for 72 h with 1 μg ml^{-1} PHA (Murex Diagnostics, Dartford). rhuIL-2 (Becton Dickinson) was added to a final concentration of 20 U ml^{-1} 18 h before infection. PBMCs (5×10^6 in experiment 1, and 1.5×10^6 in experiment 2) were incubated in 0.5 ml medium for 90 min at 37 °C with different concentrations of the indicated chemokine. Subsequently, recombinant virus (35,000 and 20,000 reverse transcriptase units for experiments 1 and 2, respectively), as well as the original chemokine concentration, were added in 1 ml. After 12 h at 37 °C the cells were washed and fresh medium without chemokine was added. After an additional 48 h at 37 °C the cells were lysed and used for measurement of CAT activity. Results are expressed as the percentage of chloramphenicol conversion observed in the absence of chemokine. The average chloramphenicol conversion for the experiments with the HXBc2 virus was 36% in HeLa cells, 20% in Cf3Th cells, and 22% in PBMCs.

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